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MECHANISMS OF VIRAL INTERFERENCE WITH MHC CLASS I ANTIGEN PROCESSING AND PRESENTATION[1]

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■ **Abstract** Viruses are ubiquitous and dangerous obligate intracellular parasites. To facilitate recognition of virus-infected cells by the immune system, vertebrates evolved a system that displays oligopeptides derived from viral proteins on the surface of cells in association with class I molecules of the major histocompatibility complex. Here we review the mechanisms counter-evolved by viruses to interfere with the generation of viral peptides, their intracellular trafficking, or the cell surface expression of class I molecules bearing viral peptides. This topic is important in its own right because the viruses that encode these proteins represent medically important pathogens, are potential vectors for vaccines or gene therapy, and provide strategies and tools for blocking immune recognition in transplantation, autoimmunity, and gene therapy. In addition, studies on viral interference provide unique insights into unfettered antigen processing and normal cellular functions that are exploited and exaggerated by viruses.

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INTRODUCTION

Immune Surveillance: A Cost Of Vertebrate Living

From the standpoint of cells, viruses are largely an unfortunate and dangerous corollary of evolution. Vertebrates responded to this threat by evolving a system capable of detecting and eradicating intracellular aliens. This system, which is based on the recognition of viral oligopeptides, exploits the absolute dependence of viruses on ribosomal production of their proteins. This did not come cheaply, as it required the evolution of the means to display peptides on the cell surface, and even more ambitiously, the development of cells capable of traveling to and distinguishing infected cells from uninfected cells and then taking effective action. Five hundred million years later (give or take 100 million years), immunologists evolved and came to term the former the major histocompatibility complex (MHC) class I antigen processing system, and the latter CD8+ T lymphocytes (T_{CD8+}).

Antigen Processing and T_{CD8+} Recognition: A Primer for Cell Biologists

Generation of Class I-Peptide Complexes The MHC describes the cluster of genes (termed H-2 and HLA, respectively, in mice and humans) devoted to processing and presenting antigens to T lymphocytes. Included are genes encoding proteins, termed MHC class I heavy chains, that display peptides on the cell surface, as well as accessory proteins devoted to the generation of peptide–class I complexes. Class I genes (along with class II genes, which encode molecules that present peptides to CD4+ T lymphocytes) are the most polymorphic known: in humans, hundreds of alleles exist at each of the three loci encoding class I heavy chains. Each allele binds a unique spectrum of peptides (Rammensee et al 1995). Binding is predominantly based on the interaction of side chains from two or three residues of the peptide with pockets present in the binding groove of the class I molecule (Madden 1995). Interactions between the ends of the groove with the peptide's main chain N and C termini also make an important contribution to the free energy of binding, so much so that peptides extending beyond the ends of the groove usually bind with affinities resulting in extremely short dwell times ($t_{1/2}$ < minutes) relative to the peptides recovered from class I molecules ($t_{1/2}$ = tens to hundreds of hours). Consequently, most antigenic peptides recovered from class I molecules are between 8 and 10 residues in length.

In generating class I ligands, cells do not discriminate between viral and self proteins. Most peptides originate from a cytosolic/nuclear pool of proteins (Yewdell & Bennink 1992). The extent to which the precursor pool consists of proteins that once achieved native status versus those that did not remains to be established. A major protease involved in the generation of antigenic peptides is the proteasome, a cylindrical, multisubunit, multicatalytic protease abundant in the nucleus and

cytosol (York & Rock 1996, Baumeister et al 1998). It is actually more accurate to use the plural because proteasomes display considerable heterogeneity in both their subunit composition and the structures that bind to the ends of the barrels. The central channel of the cylinder is only ~15 Å, meaning that substrates must be completely unfolded and threaded into the proteasome to gain access to the catalytic subunits located in the central core. Proteasomes are the primary means by which cells degrade misfolded or unwanted proteins. Most but not all substrates known to be degraded by proteasomes are conjugated to multi-ubiquitin chains. The requirement for ubiquitination in the generation of antigenic peptides is not well established and probably varies depending on the precise nature of the substrate. The proteasome is capable of generating in vitro from arbitrary substrates a number of 8- to 10-mer peptides known to be presented by class I molecules. Often, however, these are minor reaction products even after prolonged incubation. It is therefore likely that in many instances proteasomes produce an extended peptide that is further acted upon by other proteases, either in the cytosol or in the secretory pathway, to produce the very short peptides that fit optimally in the cleft.

Cytosolic peptides face a formidable topological barrier in the form of the ER membrane, which separates them from class I molecules. Cells have an intrinsically low capacity for transporting oligopeptides into the ER, and the evolutionary solution of the vertebrate immune system to this problem is TAP (for transporter-associated with antigen processing), a MHC-encoded member of the ATP–binding cassette family of membrane transporters (Elliott 1997, Momburg & Hammerling 1998). TAP consists of two homologous subunits (termed TAP1 and TAP2), each with multiple membrane-spanning domains and a cytosolically disposed nucleotide-binding domain. TAP resides primarily in the ER and ER-Golgi complex intermediate compartment.

Class I molecules consist of the aforementioned MHC-encoded heavy chains non-covalently bound to a small non-glycosylated protein, β_2 microglobulin (β_2m). The affinity of heavy chains for β_2m is greatly increased by the presence of bound peptide; in the absence of peptide, β_2m dissociates from cell surface class I molecules in minutes at 37°C. The assembly of peptide–class I complexes occurs in a intricately choreographed process in the ER (Pamer & Cresswell 1998). Class I heavy chains are typical membrane proteins with type I anchors that are cotranslationally inserted into the ER via an N-terminal leader sequence. During their insertion they are glycosylated and associate with calnexin and/or calreticulin, highly related ER-resident molecular chaperones that bind to nascent proteins with monoglucosylated N-linked oligosaccharides. Binding of β_2m to heavy chains induces the dissociation of calnexin (at least in human cells) but not calreticulin, and this complex then binds to tapasin, a MHC-encoded chaperone dedicated to class I assembly, and to another general purpose chaperone ERp57 (Lindquist et al 1998, Hughes & Cresswell 1998, Morrice & Powis 1998). This complex in turn is able to bind to TAP via tapasin (tapasin is also able to bind TAP in the absence of class I molecules and may recruit the calnexin-containing complex to TAP).

Following peptide association, class I molecules dissociate from TAP, although they maintain binding to some of the chaperones prior to their export from the ER. The entire process takes anywhere from ten minutes to hours depending on the peptide supply and probably other factors. Class I molecules are also able to bind peptides in a TAP-independent manner; the involvement of tapasin in this process is uncertain, but it is not absolutely required.

Upon their egress from the ER, class I molecules are transported via the standard Golgi complex pathway to the cell surface. A substantial number of class I molecules are delivered to the cell surface without high–affinity ligands, and β_2m rapidly exchanges with plasma β_2m (present at 1.5 μg/ml in humans), thereby maintaining class I molecules in a peptide-receptive state for $\sim$30 min until heavy chains irreversibly denature and are either degraded in endosomes/lysosomes or shed. Peptide-bearing class I molecules are also internalized and degraded but at a much lower rate.

T_{CD8+} Recognition of Viral Antigens T_{CD8+} populations in individuals are made up of millions of clones, with each clone expressing a unique form of the T cell antigen receptor (TCR). Each TCR expressed by individual clones walks a fine thermodynamic line because T cell development in the thymus and survival in the periphery demands a certain threshold affinity for self–class I molecules bearing generic peptides, but less than the affinity required for triggering (Kiselow & von Boehmer 1995, Stockinger 1999). Recognition of foreign antigens is based on binding to a class I molecule bearing a foreign peptide at an affinity higher than this threshold, but still not very impressive (compared with immunoglobulins), with a K_D in the range of 10^{-4} to 10^{-6} M (Davis et al 1998). Due, however, to the enormous increase in avidity afforded by expressing multiple copies of the receptor (tens of thousands TCRs/cell), T_{CD8+} can be extremely sensitive despite this low affinity, with many virus-specific clones requiring on the order of 10–100 complexes per target cell surface for triggering.

It is important to distinguish between two types of T_{CD8+} triggering: the activation of naïve T_{CD8+} to induce proliferation and synthesis of effector molecules with anti-viral activity and the triggering of T_{CD8+} by virus-infected cells to induce release of the effector molecules. The former can be accomplished only by cells that express the proper costimulatory molecules. These cells are the so-called professional antigen-presenting cells (APCs), whereas the latter requires only the appropriate peptide class I complex. Professional APCs have the capacity to generate peptides from exogenous antigens, i.e. viral proteins released from infected cells (Yewdell et al 1999). This may have evolved as a means for the immune system to prevent viruses from using the mechanisms described below to block activation of T_{CD8+} responses. In any event, the result is that given a viral mechanism to block processing of biosynthesized antigens in infected cells (endogenous antigens), there is still a good chance there will be a T_{CD8+} response to the antigen (depending on the extent that the exogenous pathway can create a sufficient number of determinants on professional APCs to activate naïve T_{CD8+}). Therefore, the

presence of a T_{CD8+} response to a given viral determinant cannot be equated with the presentation of the determinant by infected cells.

Immunodominance A basic feature of T_{CD8+} responses to viruses is that only a tiny fraction (1 to 20 or so) of the thousands to hundreds of thousands of different peptides (depending on the complexity of the virus) generated by infected cells induce T_{CD8+} responses (Yewdell & Bennink 1999). This is due to a number of factors, the most important of which is that only ~0.5% of peptides of the correct length bind to any given class I molecule allele (see below) with sufficient affinity ($K_D > 5 \times 10^{-7}$) to produce a sufficient number of complexes to trigger T_{CD8+} activation. Second, the TCR repertoire can distinguish as foreign only about half of the viral peptides that bind to class I molecules above this threshold affinity. Third, the antigen processing machinery is limiting for production of ~80% of the determinants that can potentially bind to class I molecules, mostly because of limitations in proteolytic liberation of determinants. Fourth, a strong T_{CD8+} response to a given determinant suppresses responses to other determinants (this is known as immunodomination). Fifth, to maximize viral replication, many viruses shut down the synthesis of host proteins, including class I molecules. If the supply of class I molecules is limiting, then determinants from viral proteins expressed late in the infectious cycle will have no means of presentation to T_{CD8+} (this applies only to cases in which T_{CD8+} activation requires presentation of endogenous antigens by professional APCs). Finally, and most relevant to this review, viruses may selectively interfere with the presentation of some viral gene products or determinants, leaving the immune system to focus on those determinants left unscathed.

VIRAL INTERFERENCE WITH ANTIGEN PROCESSING

Viral Strategies for Avoiding T_{CD8+} Surveillance: General Considerations

Determinant-Specific Blockade: MHC Polymorphism Given the limited number of determinants recognized by T_{CD8+} and the prodigious abilities of viruses to mutate, the simplest means for viruses to avoid T_{CD8+} recognition is to mutate their proteins to block either antigen processing, class I binding, or TCR discrimination from self determinants by mimicking self peptides (McMichael & Phillips 1997). That this seldom occurs is probably due to class I polymorphism. Each individual expresses up to six different class I alleles (there are three class I loci per HLA), and for each locus, hundreds of alleles exist at appreciable frequencies in the world-wide population (alleles at the three loci are collectively referred to as allomorphs because their gene products are highly similar in form and function). Most of the amino acid differences between allomorphs cluster in regions of the binding site that dictate peptide specificity. Consequently, each allomorph presents a unique spectrum of peptides to T_{CD8+}. Any viral mutant that escapes T_{CD8+}

immunity in a given individual is unlikely to maintain the selective advantage in its next victim—indeed, it is thought that viruses provide the major selection pressure for maintaining polymorphism in class I genes in populations. The value of MHC polymorphism can perhaps best be judged by its high cost. Since the immune system does not know which allomorphs will be expressed by any given individual, the T_{CD8+} repertoire cannot be hardwired, necessitating thymic selection instead.

In addition to peptide binding, class I allomorphs exhibit important differences in their assembly in the ER and stability on the cell surface. These differences result from amino acid alterations both in and out of the binding site. The bases for these effects are usually poorly understood. For the purposes of the present discussion, the practical result is that allomorph specificity does not necessarily mean that a viral gene product acts by directly interacting with class I molecules. For example, blocking TAP-mediated peptide transport has a much less severe effect on allomorphs that preferentially bind peptides derived from signal sequences, which usually gain access to class I molecules through the translocon.

Broader Strategies for Blocking T_{CD8+} Recognition Largely stymied by MHC polymorphism, and given geologic time to ponder the problem, a number of viruses evolved proteins that exert a more general blockade to antigen processing or presentation (summarized in Figure 1). All the known proteins with such functions are encoded by viruses that cause chronic infections. With the exception of human immunodeficiency virus (HIV), all are DNA viruses with medium to large genomes, so that constraints of packaging genomes into virions do not severely limit the acquisition of new genes. This implies first, that these genes are not of much use in acute infections and, second, that the most fertile grounds for finding novel viral genes that interfere with T_{CD8+} recognition are large viruses that cause chronic infections.

Before discussing these strategies, it is important to note that assigning function to a gene product is an exercise fraught with danger, since function must ultimately be assessed on an evolutionary basis. While it is clearly simpler to ascertain the functions of viral gene products that interfere with host immunity relative to cellular gene products, it still poses great technical challenges when the gene product is encoded by a human virus. Dissecting the function of immunomodulatory genes can be assessed with any degree of precision only in mice, where it is possible to infect under controlled conditions and measure the responses of individual arms of the immune response. Even given a human virus that can infect mice (or can be made to do so by generating transgenic mice expressing the receptor, etc), the infection always differs from the natural infection in humans, usually considerably. Human class I molecules can be expressed in mice, but the antigen-processing machinery remains of mouse origin, of course, and T_{CD8+} are but one part of an immune response that consists of numerous other cell types, all of which are expected to behave at least slightly differently in mice and humans. This is not to say that assigning function to immunomodulatory molecules is a hopeless endeavor, but that it ought be considered a work in process. Not to belabor the

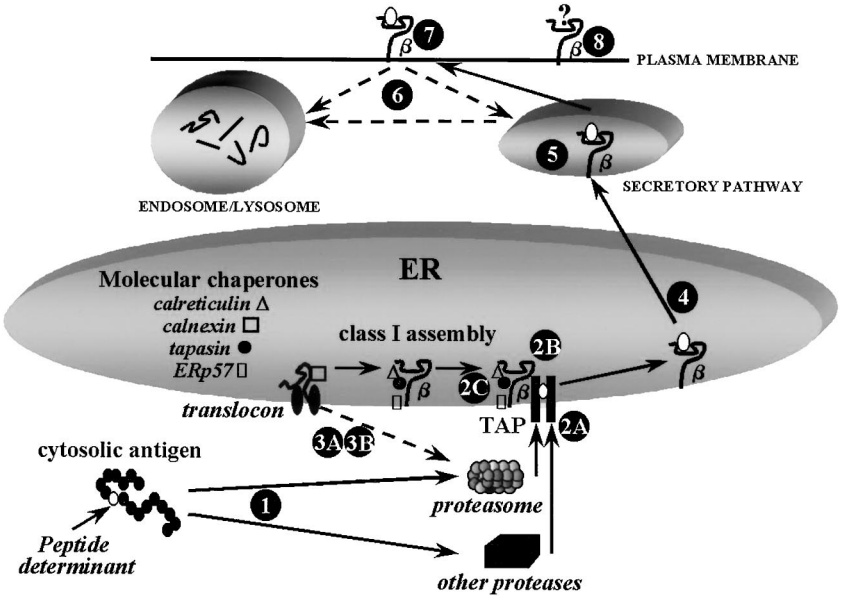

Figure 1 Viral Interference with Antigen Presentation: Greatest Hits. The sites of action of viral proteins that interfere with antigen processing are indicated by the numbers. Most peptides presented by class I molecules are produced from a cytosolic pool of proteins from the action of proteasomes and probably other proteases (black box). EBV-encoded EBNA1 possesses a long Gly-Ala repeat that interferes with peptide generation, probably by blocking proteasome digestion (1). In contrast to this *cis*-acting effect, hCMV-encoded pp65 acts in *trans*- to block the generation of peptides from the early hMCV viral protein IE (but not other viral proteins), but the precise mechanism remains to be established. Transport of cytosolic peptide into the ER predominantly occurs via TAP; this is blocked on the cytosolic side by HSV-encoded ICP47 (2A) and the lumenal side by hCMV-encoded US6 (2B). Class I molecules are assembled in the ER in a complex process involving multiple general purpose (calreticulin, calnexin, Erp57) and specialized (tapasin) molecular chaperones. Newly synthesized class I molecules are redirected to the cytosol for proteasomal destruction by hCMV-encoded US2 and US11 (3A), and probably by HIV-encoded Vpu (3B), which may utilize a different mechanism in which active proteasomes are required for export. Class I molecules with chaperones bind to TAP via tapasin, an interaction interfered with by adenovirus-encoded E19 (2C). E19 also retains class I molecules in the ER (4) as does hCMV-encoded US3. Class I molecules can be retained in the ERGIC, as accomplished by mCMV-encoded gp40 (5), or targeted from the ER to lysosomes for destruction (6) by mCMV gp48. Lysosomal targeting of class I molecules can also occur from the *trans*-Golgi complex or from the cell surface (7), as accomplished by HIV-encoded Nef. Dotted lines refer to difficulties in establishing the routing of proteins between the *trans*-Golgi complex, cell surface, and endosomes. Finally (8), complexes of viral proteins (mCMV-encoded gp34) with class I molecules formed in the ER may be expressed on the cell surface, for an uncertain purpose.

point, but function should probably be thought of as proposed function, or at best, likely function.

As will become apparent, it has not been difficult for viruses to evolve mechanisms for blocking antigen presentation. This comes as no surprise because it is far easier to destroy than to create. The immune parry to this thrust is to monitor cells for the absence of class I molecules, which is accomplished by NK cells, another type of immune cell with a T_{CD8+}-like anti-viral armamentarium (Lanier 1998, Moretta et al 1996). Although it is beyond the scope of this review to discuss how viruses deal with NK cells, it is important to consider viral blockade of antigen presentation as but one front in the larger conflict between virus and host. The difficulties of avoiding both T_{CD8+} and NK recognition is the sort of thing that keeps viruses awake at night and must must limit the evolution of viral genes that block antigen presentation.

Why This is Interesting, Important, or Both Obviously, understanding how a given virus interferes with antigen presentation is needed to understand virus-host interaction, with the pragmatic goal of interfering on behalf of the host (us). Viral proteins with defined means of blocking antigen presentation have practical applications for both bench and bedside. First, they can be used as tools to mechanistically determine pathways used for presentation in vitro and in vivo. Second, they have considerable therapeutic potential as immunosuppressive agents in transplantation, gene therapy, and autoimmunity.

Less directly, studies of viral interference can give important insight into unappreciated aspects of antigen processing and presentation and, particularly for many readers of this volume, more general areas of cell biology. For example, the sequence for ER retention was identified while studying the adenovirus E19 glycoprotein (Jackson et al 1990, Nilsson et al 1989); the first protein inhibitor of an ABC transport protein is the HSV ICP47 protein (Hill et al 1995, Früh et al 1995); and the ER to cytosol pathway of proteasomal destruction was first identified in mammalian cells studying cytomegalovirus US2 and US11 proteins (Wiertz et al 1996a,b).

Viral Strategies for Avoiding T_{CD8+} Surveillance: Specific Targets

Preliminaries This review deals exclusively with viral interference with cellular proteins involved with antigen processing and presentation. We would be remiss, however, if we neglected to mention the alternate stratagem of blocking expression of antigen-processing genes, which accomplishes the same thing, albeit less rapidly. This is well documented for adenoviruses (which exploit common promoter usage to decrease expression of multiple components of the antigen-processing system), and we direct readers to recent reviews (Ehrlich 1997, Sparer & Gooding 1998).

We need also mention that viruses have sophisticated strategies for modifying immune responses by interfering with the messengers that regulate immune cell

trafficking, activation, and effector functions, and the sensitivities of infected cells to immune effector functions (see recent reviews; Spriggs 1996, McFadden et al 1998).

Given a viral gene product thought to interfere with antigen processing, two basic approaches can be used for characterization: The gene product can be studied in isolation by expressing it as a transgene using various vectors, or the effects of deleting or modifying the gene product in the context of the source virus can characterized. The limitation of the first approach is that the activity of the gene product may be modified by other viral gene products or indirect effects of viral infection, whereas the second approach cannot distinguish direct versus indirect effects of the deleted gene product. Thus a thorough understanding requires pursuit of both approaches, particularly in situations in which viruses encode multiple gene products that interfere with antigen processing.

Breaking Up Is Hard to Do: Blocking Peptide Generation The two known examples of blocking peptide liberation occur with members of the *Herpesviridae*, the undisputed champions among virus families of blocking antigen presentation. The better characterized example occurs with Epstein Barr virus (EBV) nuclear antigen 1 (EBNA1). EBNA1 is the only viral protein known to be expressed by all latently infected B cells, presumably because it is required to maintain latency. Based on its indispensability, EBNA1 may be subject to unique T_{CD8+}-mediated selection pressure to block its presentation. It is not possible to detect presentation of EBNA1-encoded peptides by cells synthesizing amounts of the protein at levels more than sufficient for presentation of determinants from other proteins (Levitskaya et al 1995). EBNA1 contains an unusual 238-residue domain consisting solely of Gly and Ala residues. Transfer of this region to other proteins hinders the presentation of class I–associated peptides (Levitskaya et al 1995), and removal of the sequence enables the generation and presentation of EBNA1 peptides to T_{CD8+} (Blake et al 1997).

The effect of the Gly-Ala sequence on ubiquitin-conjugation and proteasome degradation was examined using a cell-free rabbit reticulocyte system (Levitskaya et al 1997). Following in vitro translation, EBNA4 (a control EBV protein whose presentation to T_{CD8+} is blocked by proteasome inhibitors) was ubiquitinated and degraded by proteasomes, while EBNA1 was neither ubiquitinated nor degraded by proteasomes. Removal of the Gly-Ala repeat increased the ATP-dependent degradation of EBNA1, as predicted, but the repeat-free protein could not be shown to be ubiquitinated, nor was the degradation blocked by the proteasome inhibitor that blocked EBNA4 degradation. Insertion of the 239-long Gly-Ala repeat into EBNA4, or even a 17-residue alternating Gly-Ala sequence, prevented EBNA4 degradation, an effect that occurred independently of the position of the insert in the protein. There was no discernible effect on ubiquitination, suggesting that the blocking effect occurred following ubiquitination.

The other example of viral inhibition of peptide generation is somewhat less well defined and its in vivo relevance even more uncertain. The human cytomegalovirus (hCMV) immediate early (IE) protein is presented to T_{CD8+}-specific for the protein

when IE is expressed by recombinant vaccinia virus (rVV) but not by hCMV itself (Gilbert et al 1993, 1996). This effect occurs for an impressive number of IE peptides presented in association with different HLA allomorphs, and presentation of these determinants in rVV infected cells can be blocked by co-infection with a rVV-expressing the hCMV pp65 gene product. Expression of pp65 has no discernible effect on the generation of antigenic peptides from proteins other than IE, including another hCMV protein. Moreover, pp65 itself is presented to specific T_{CD8+} when expressed by hCMV or by VV, at an apparently typical efficiency. pp65 is abundant in virions, and by comparison with a virus lacking pp65, it could be shown that sufficient virion pp65 is delivered to cells (at least in vitro) to block antigenic peptide generation from IE. pp65 is a Ser/Thr kinase, and this activity is required both for the phosphorylation of IE and for blocking peptide generation from IE, as determined by expression of both wild-type and a truncated version of pp65 lacking the 58 C-terminal residues.

The observations that pp65 blocks presentation of IE and not other proteins and that multiple determinants presented by different allomorphs are blocked strongly implicate proteolysis as the site of the pp65 blockade. Although the metabolic stability of IE was not detectably affected by expression of wild-type or truncated pp65, this was determined using a IE-specific mAb, and it is possible that pp65 interferes with the proteolysis of forms of IE that do not react with the mAb. The involvement of the proteasome in the presentation of IE peptides has not been examined and should be studied in the future. If presentation of IE peptides is proteasome dependent, it would imply that pp65 decreases IE ubiquitination or other means of targeting IE to proteasomes. Alternatively, pp65 could modify the degradation of IE by non-proteasomal proteases.

Given the presentation of proteins (including pp65) from input virions (Riddell et al 1991), the biological relevance of the pp65-IE blockade cannot be assumed (although relevant scenarios can be envisaged: e.g. the amount of pp65 delivered is sufficient to block IE presentation, but insufficient for recognition by pp65-specific T_{CD8}). However, it is clear that this system can provide tremendous insight into the nature of substrates acted on by peptidogenic proteases, as well as the more basic issue of how proteins are targeted for degradation.

Stop! in the Name of Love (or* Herpesviridae*): Blocking TAP-Mediated Peptide Transport into the ER Two features of TAP make it an attractive target for viruses intent on blocking antigen presentation. First, TAP is dedicated to antigen processing, and blocking its function will have little if any effect on cell metabolism and viral replication. Second, TAP is involved in the presentation of upward of 90% of peptide presented by most class I allomorphs.

Two viral gene products are known to exploit this vulnerability. The first discovered and best characterized is HSV ICP47, a 87 residue protein expressed very early in the infectious cycle that is dispensable for in vitro replication. It was initially shown to prevent the assembly of class I molecules (York et al 1994). The protein sequence lacks an ER insertion sequence, and when overexpressed, ICP47

is present largely in the cytoplasm and nucleus, pointing to an indirect effect on class I assembly (York et al 1994). It was subsequently shown that ICP47 blocks TAP–mediated peptide transport and is tightly bound to the TAP1-TAP2 complex, as demonstrated by coprecipitation in a detergent [Triton X100 (TX100)] that interfere with weak protein-protein interactions (Früh et al 1995, Hill et al 1995). ICP47 fails to inhibit ATP binding to either TAP subunit, and similar to TAP-binding peptides, is unable to bind to either TAP subunit expressed in the absence of the other (Hill et al 1995). This, and observations that ICP47 competes with peptide binding to TAP without being transported into the ER (inferred from its failure to acquire Asn-linked oligosaccharides, the assay used to measure peptide transport into the ER) (Ahn et al 1996b), suggest that ICP47 interacts with the cytosolic domain of TAP in a manner that prevents peptide binding. ICP47 binds to TAP with a K_d of $\sim$50 nM, or about tenfold higher affinity than the highest affinity peptides.

Boiling of ICP47 produced by bacteria does not affect its activity (Ahn et al 1996b), and full activity is exhibited by a synthetic peptide version of the protein (Galocha et al 1997). The synthetic peptide behaves in aqueous solution like a random coil. These findings indicate that ICP47 activity does not require much, if any, stable folding. Consistent with this conclusion, synthetic peptides corresponding to fragments of ICP47 as small as residues 3–34 exhibit nearly complete blocking activity (Beinert et al 1997).

The high activity of truncated versions of ICP47 prompts the question of why the other residues have been maintained by HSV. There are two general possibilities.

1. Residues 35–89 are required to prevent the misfolding of the biosynthesized protein (an exceptional case where misfolding would be the acquisition of structure) or its degradation in vivo (given its extended structure, ICP47 would seem to be a sitting duck for proteolysis). One area for future study is to characterize the fate of biosynthesized versions of the truncated ICP47s. A somewhat similar experiment has been conducted by nature. The ICP47 gene of HSV2 has a 13-base-pair deletion in the region of the gene encoding residue 59 of HSV1 ICP47, which has resulted in a frame shift (Galocha et al 1997). The gene product is a similar length, however, suggesting that polypeptide length may be important for the activity of biosynthesized ICP47.
2. Residues 35–89 are required for unrelated activities of ICP47 (although the differences between the HSV1 and HSV2 ICP47 suggest that this is would not be the sole factor in maintaining the residues in viral evolution).

A clue to the mechanism of ICP47 blockade of TAP is that it exhibits a high species selectivity. Both HSV1 and HSV2 ICP47 inhibit ape, monkey, pig, dog, and cow TAP and have little effect on mouse, rat, guinea pig, or rabbit TAP (Jugovic et al 1998). The affinity of ICP47 to human TAP is approximately 100-fold higher than for mouse TAP (Ahn et al 1996b). Given that TAPs from these species probably overlap considerably in the specificies of their peptide–binding sites, this

suggests that ICP47 makes thermodynamically important contacts with residues not strictly involved in peptide binding that serve to enhance its affinity. Indeed, ICP47 may not contact the peptide-binding site at all. In contrast to peptides, which increase the degree of cross-linking of TAP1 and TAP2 subunits following exposure to a homo-bifunctional cross-linker, ICP47 greatly reduces the recovery of cross-linked TAP (Lacaille & Androlewicz 1998). This suggests that ICP47 acts by modifying TAP conformation in a way that negatively impacts peptide binding. Although ICP47 appears to be a competitive inhibitor of peptide binding, it is difficult to distinguish true competition between ligands for the same site versus competition for stabilizing alternative conformations that disfavor the binding of the competing entity.

ICP47 inactivates TAP expressed in yeast or insect cells (Urlinger et al 1997, Beinert et al 1997), demonstrating that special factors expressed by antigen–presenting cells are not required for its interaction with TAP. It remains to be formally established, however, whether ICP47 binds to TAP in the absence of other cellular proteins. Based on the acquisition of an α-helical structure by a synthetic peptide (residues 1–53) of ICP47 (in the presence of membrane mimetic solutions or membrane vesicles composed of negatively charged lipids), as well as on calculated secondary structure predictions, residues 3–13 and 35–43 were proposed to form α-helices that associate with the ER membrane, thereby favoring association with TAP (Beinert et al 1997). If membrane association occurs, it may follow binding to TAP, however, because as noted above, in the absence of TAP, ICP47 does not detectably associate with cellular membranes (York et al. 1994).

Evincing admirable creativity, *Herpesviridae* evolved another means of blocking TAP. The hCMV US6 protein is a 186-residue glycoprotein with a type I membrane anchor that localizes to the ER. US6 blocks TAP-mediated peptide transport, but unlike ICP47, it does not interfere with peptide binding to TAP (Ahn et al 1997, Hengel et al 1997). The US6 lumenal domain is required for both ER retention and TAP blockade, whereas the transmembrane and cytosolic domains are dispensable for both functions (Ahn et al 1997). US6 binds to TAP complexed with class I molecules, tapasin and calreticulin (Hengel et al 1997). US6 is able to block TAP function when it is translated in vitro into TAP-containing microsomes, even those derived from cells lacking class I molecules or functional tapasin (Hengel et al 1997), demonstrating that US6 probably binds directly to TAP and not through an intermediary. It remains to be established whether the ER retention of US6 is based solely on binding to TAP: Expression of US6 in excess of TAP does not result in ER export of US6, but this could be due either to intrinsic ER localization signals or to transient interaction with TAP (Ahn et al 1997). The US6 cytosolic tail possesses a di-Arg sequence that may contribute to ER retention (Lehner et al 1997), but the tail is not required for ER retention, at least not in TAP-expressing cells (Ahn et al 1997).

US6 presumably acts by inducing a conformational alteration in TAP, but other than blocking peptide binding, the nature of this alteration is undefined. It remains for future studies to determine whether (*a*) assembly of TAP is required for binding

of US6, (*b*) TAP nucleotide binding is blocked by US6, or (*c*) US6 binding resembles ICP47 in reducing chemical cross-linking of TAP1 and TAP2.

Never Say Good-Bye: Retaining Class I Molecules in the Secretory Pathway From the perspective of human discovery, the oldest known viral interfering protein is the adenovirus E19 protein. The strong interaction of E19 with MHC class I molecules was discovered at a time (mid 1980s) when the reigning paradigm dictated that T_{CD8+} recognize intact proteins, leading to the (now) ironic interpretation that adenovirus-specific T_{CD8+} recognize E19 in association with class I molecules (Pääbo et al 1983, 1985; Signas et al 1982). Several years later it was recognized that E19 not only is retained in the secretory pathway, but in so being prevents class I expression. It was correctly surmised that this represented the first example of a virus interfering with antigen presentation (Andersson et al 1985, Burgert & Kvist 1985), which was later demonstrated directly (Andersson et al 1987, Cox et al 1990). E19 is by far the best characterized viral interference protein. Even so, significant gaps in understanding remain, and recent findings provide an unexpected twist in the E19 story.

E19 is a 142-residue glycoprotein with a type I membrane anchor. Its 15 residue tail C-terminal tail was the prototype for retention of ER membrane proteins mediated by cytosolic domains (Jackson et al 1990, Nilsson et al 1989), now believed to be based on coatomer binding to a di-Lys motif (Cosson et al 1998). E19 is probably a multimer because a subpopulation migrates as dimers in SDS-PAGE under nonreducing conditions (Cox et al 1990, Sester & Burgert 1994). E19 binds tightly to class I molecules via its lumenal domain in a manner independent of glycosylation of either component (Burgert & Kvist 1987), and binding appears not to require specialized cofactors, as it occurs in insect cells (Lévy & Kvist 1991) or in a cell-free system with purified class I molecules and glycoproteins purified from E19–expressing cells (Pääbo et al 1986). Folding of the E19 lumenal domain into a class I binding conformation requires disulfide bond formation between two pairs of Cys residues conserved among E19s expressed by different adenovirus serotypes (Sester & Burgert 1994, Deryckere & Burgert 1996). Both E19 and class I molecules are able bind to each other rapidly following their biosynthesis, as both molecules are recovered at (or near) maximal amounts following 10 min pulse radiolabeling with [^{35}S]-Met using antibodies specific for either component (Cox et al 1990). E19 is capable of binding to class I α-chains synthesized from microinjected mRNA in the absence of β_2m in *Xenopus* oocytes (these cells may express endogenous β_2m, but regardless, α–chains behave as if they are not associated with β_2m) (Severinsson & Peterson 1985). E19 binding to class I molecules can result in the absence of β_2m from the complex (Kämpe et al 1983, Cox et al 1990), although β_2m is often present in E19-class I complexes, and the extent to which E19 binding to α-chains precedes or precludes β_2m association remains to be established. Now that class I folding is understood in more detail, it will be of interest to examine the conformational state of class I molecules bound by E19 and the possible presence of accessory molecules in the complex.

E19 is highly selective for binding to different class I allomorphs. Even weak binding to HLA molecules, at least as determined by coprecipitation from TX100 extracts, results in delayed transport from the ER (Beier et al 1994). Binding to some mouse class I allomorphs is not detected under these conditions, and their transport from the ER is not slowed (Cox et al 1990, Beier et al 1994), supporting the physiological relevance of coprecipitation studies. Using chimeric class I molecules produced from E19-binding and nonbinding mouse allomorphs, residues that influence association with E19 map to the $\alpha 1\alpha 2$ domains of class I molecules (Burgert & Kvist 1987). Consistent with this finding, some mAbs specific for residues in the α-helices of HLA molecules are unable to coprecipitate E19 with class I molecules, an effect that can be attributed to antibody displacement of E19 and/or conformational alterations induced by antibody binding (Flomenberg et al 1994). The three mAbs with this property (of a panel of six tested) are thought to interact with residues in the α–helices located above the portion of the groove that interacts with the peptide N terminus. However, it appears that E19 binding does not block peptide binding to class I molecules. This is inferred by the inability of E19 to block antigen presentation in association with H-2K^d molecules when six residues at the C terminus that are required for ER-retention are removed (Cox et al 1991). This molecule co-precipitated similar amounts of H-2K^d as wild-type E19, and functionally competed with E19, suggesting that it binds to H-2K^d in an indistinguishable manner. This evidence, while compelling, is indirect, and it remains to be rigorously established whether class I molecules associate with E19-containing peptides (see below).

Looking at the E19-class I complex from the other side, the diversity among E19s from three (of the five) human adenovirus serotypes that express E19 is remarkable; only 24 of the 100 residues in the lumenal domain are conserved (Deryckere & Burgert 1996). Obviously, proteins with widely divergent sequences can form highly similar structures (e.g. MHC class I and class II molecules), and despite the 25% homology, it is expected that the structures are highly similar, particularly because the Cys residues that participate in disulfide bonds are conserved.

Such indirect approaches to understanding the structural basis for the interaction between E19 and class I molecules are well into the nether regions of diminishing returns relative to the efforts required. Cutting this Gordian knot entails the X-ray crystallographic solution of the complex, which should stimulate a renewed round of mutagenesis/structure/function studies.

The mechanism of E19 blocking antigen presentation may be more complicated than simply retaining peptide-bearing class I molecules in the ER. Immunoprecipitation of H-2K^d results in the coprecipitation of a membrane glycoprotein doublet migrating in SDS-PAGE with 110/100 kDa, with a lumenal domain of 32 kDa as determined by protease digestion of microsomes (Feuerbach & Burgert 1993). Coprecipitation of p110/p100 with H-2K^d is greatly enhanced by expression of E19 and is further enhanced by glucose starvation, suggesting that p110/100 is an ER chaperone involved in glycoprotein folding. Most interestingly, addition

of a H-2K^d binding peptide results in the release of p110/100 from H-2K^d, suggesting that it binds to peptide-free H-2K^d molecules, and by inference, that E19 expression slows class I acquisition of peptide.

The latter point is consistent with findings that E19 lacking the six-C-terminal residues required for ER-retention (ΔE19) slows the export of class I molecules from the ER (Cox et al 1991). When the basis for this was explored, both E19 and ΔE19 were found to block the association of class I molecules with TAP (Bennet et al 1999). Apparently this is not accomplished simply by E19 binding to class I molecules, as might be expected, but by the binding of E19 to TAP, which occurs independently of class I molecules and tapasin. As E19 does not detectably affect the association of tapasin with TAP, it appears that E19 blocks the tapasin-mediated binding of class I molecules to TAP. Given an efficient means of blocking antigen presentation (ER retention), why should E19 bother to block class I association with TAP? The answer may lie in the variability of E19 association with different human allomorphs: Blocking TAP association can be seen as a means of adenovirus to counter MHC polymorphism, at least partially. These findings were made with E19 derived from a single serotype; it will be interesting to determine whether E19 gene products from the different adenovirus subgroups behave similarly.

The US3 gene product of hCMV is a 187 residue integral membrane glycoprotein (Jones et al 1996, Ahn et al 1996a). The protein is degraded with a $t_{1/2}$ of ~30–60 min, and US3 that escapes degradation appears to be slowly exported from the ER, as inferred from maturation of the single N-linked oligosaccharide measured by resistance to sugar cleavage by endoglycosidase H (endo H) (an imperfect measure of glycoprotein transport because N-linked oligosaccharides are not always modified during transit through the Golgi complex). Class I molecules expressed in the presence of US3 are not degraded more rapidly, however, suggesting either that US3 associates transiently with class I molecules, or that US3 bound to class I molecules is spared the degradative fate of uncomplexed US3. US3 coprecipitates with conformed HLA class I molecules: This requires the use of digitonin (and not NP40), a detergent highly similar to TX100, to solubilize membranes, which may indicate that US3 binds to a component of the class I assembly complex and not class I molecules themselves. Arguing against this, however, is the observation that the effect of US3 appears to be allomorph selective (Jones et al 1996). Expression of US3 slows but does not prevent class I acquisition of high–affinity peptides, as determined by recovery of class I molecules with a conformationally sensitive mAb after incubation of detergent extracts at 37°C (Ahn et al 1996a). This latter finding suggests that US3 may interfere with class I association with tapasin/TAP as does E19.

In addition to its effect on class I expression, US3 and truncated versions have been reported to activate transcription from the HSP70 promoter. The relationship between these apparently disparate functions of US3 remains to be established (Tenney et al 1993), but scenarios could be imagined in which HSP70 upregulation modifies or retards peptide generation in the cytosol, or in which other cellular genes involved in antigen processing are dysregulated.

Mouse CMV (mCMV) gp40 is a 378-residue integral membrane glycoprotein with a type I anchor whose expression results in the accumulation of class I molecules in the *cis*-Golgi complex/ER Golgi complex intermediate compartment (ERGIC) (Ziegler et al 1997). Similar to US3, the protein is degraded with a $t_{1/2}$ of ~2 h, and the undergraded material becomes endo H resistant, while class I molecules persist in an endo H-sensitive state for prolonged periods and apparently are not degraded. gp40 binds to a wide variety of mouse class I allomorphs, but not to human allomorphs, a distinction that is maintained in human cells. This strongly suggests a direct interaction between gp40 and mouse class I molecules, but the interaction has not been detected, even in digitonin extracts. The cytoplasmic tail of gp40 contains the YRLV motif often associated with targeting to lysosomes, and as expected, the tail is not required for retention of gp40 in the ERGIC. These findings suggest a model in which gp40 transiently interacts with class I molecules in the ERGIC, with free gp40 being slowly exported from the ERGIC and targeted to lysosomes for destruction. The functional significance of gp40 trafficking to lysosomes remains to be elucidated.

Return to Sender: Shipping Class I Molecules Back to the Cytosol When it comes to devising creative ways of blocking antigen presentation, hCMV is a genius. Perhaps the most remarkable mechanism devised by hCMV is the proteasome-mediated destruction of newly synthesized class I molecules. This is achieved by either of two hCMV glycoproteins, US2 or US11, which are highly related evolutionarily to each other and to US3 (US 2 and US11 share a 21% sequence identity and 45% sequence similarity; US2 and US3 share a 23% sequence identity and 56% sequence similarity) (Ahn et al 1996a). *US2* and *US11* genes encode, respectively, sequences of 199 and 215 residues: Each is thought to be a type I anchored integral membrane protein.

In cells expressing US11 from a transfected gene, human heavy chains are destroyed with a $t_{1/2}$ of ~1 min (Wiertz et al 1996a). Glycosylation of class I molecules is not required for US11-mediated degradation. Proteolysis is blocked by proteasome inhibitors, and class I molecules spared destruction are present almost entirely in cytosolic fractions of homogenized cells, suggesting that US11 induces the retrograde transport of nascent class I molecules to the cytosol. Consistent with this, class I molecules are deglycosylated (presumably by a peptide *N*-glycanase that is believed to reside in the cytosol), and deglycosylated class I molecules can be recovered in association with proteasomes (Wiertz et al 1996b). US11 does not prevent the formation of an early class I folding intermediate reactive with a mAb (W6/32) that binds to a discontinuous determinant on native class I molecules, but these folding intermediates are also ruthlessly destroyed.

To date, only class I molecules are known to be affected by US11. Although the number of additional proteins examined is small, it has been suggested that the survival of transfected cells expressing US11 (and US2) precludes wholesale destruction of ER-targeted proteins. US11 only weakly coprecipitates with class I molecules, possibly due to the transience of the interaction (Ahn et al 1996a). Given the relatively long $t_{1/2}$ of US11 (~2 h), this observation is consistent with the

idea that US11 is a class I serial killer. The binding to class I, however transient, may contribute to the retention of US11 in the ER (as determined by immunofluorescence) (Ahn et al 1996a) and immuno-EM (Wiertz et al 1996a), as US11 has no other obvious means of achieving ER-retention.

US2 causes a similar translocation of class I molecules into the cytosol with attendant proteasome-dependent destruction, although the degradation appears to occur more slowly, with a $t_{1/2}$ of ~30 min (Wiertz et al 1996b), making it clear that translocation into the cytosol can occur well after insertion into the ER has occurred. In this case, glycosylated class I molecules could be detected in the cytosolic fraction, providing evidence that deglycosylation follows translocation from the ER. Reducing cellular ATP levels results in a blockade of ER-to-cytosol transport, enabling cells to accumulate mAb W6/32-conformed molecules in the ER. Treatment of US2–expressing cells with a reducing agent slowed degradation of α-chains and also blocked class I folding detected by binding to the W6/32 mAb. Removal of the reducing agent enabled the folding of α-chains, which were then degraded, suggesting either that folded class I molecules are the preferred substrate or that US2 activity also requires refolding.

When US2-specific antibodies were used to recover US2-class I complexes, it was found that US2 associates with both unfolded and W6/32-reactive class I molecules; in the former case, the recovered US2 was non-glycosylated, in the latter it was glycosylated. These findings indicate that US2 accompanies heavy chains to the cytosol and are consistent with the idea that US2 acts exclusively on conformed class I molecules, but does not eliminate the possibility that unfolded class I molecules are also degraded (which can be easily tested using β_2m-deficient cells). Both α–chains and US2 were present in complexes isolated from digitonin extracts of proteasome inhibitor-treated cells with antisera raised to Sec61β or Sec61β, constituents of the translocon complex. Only deglycosylated α-chains were recovered, whereas US2 was mostly deglycosylated. These findings suggest that the translocon is involved in the ER–to–cytosol pathway. They also imply either that cytosolic deglycosylation occurs while the proteins are in the process of translocation or that deglycosylation can also occur in the ER lumen.

The central question posed by these remarkable findings is how US2 or US11 direct class I molecules to the cytosol. The observation that the half lives of US2 and US11 are longer than class I molecules suggests that class I-US2/11 complexes are preferentially selected for retrograde translocation. The original study clearly demonstrated that US2 or US11 is not necessary for the retrograde translocation of class I molecules, which occurs in their absence, albeit at a much lower rate. That these proteins are not creating a novel pathway but instead exaggerate a normal cellular process has been confirmed in numerous systems: Much of what was thought to represent degradation in the ER of misassembled proteins (termed ERAD for ER-associated degradation) represents retrograde translocation and proteasomal degradation. Indeed, retrograde translocation is even thought to represent the major means of generating antigenic peptides from ER-targeted protein (Bacik et al 1997, Mosse et al 1998).

Many other questions remain.

1. Does the protein really pass through the translocon on the way to the proteasome?
2. If so, how can US2/11 remain associated with class I, since the translocon is thought to accommodate only extended polypeptide chains?
3. If so, are there in and out translocons, or only a two-way translocon?
4. How does non-ubiquitinated class I or US2 associate with proteasomes? Is it part of a larger complex involved in ubiquitination/degradation, or is class I degradation ubiquitination independent?
5. What is the basis for recognition of class I molecules by US2 and US11? Tools that may be of use are the differential degradation of mouse class I allomorphs by US2 (Machold et al 1997) and the failure of US2 or US11 to impair biogenesis of HLA C and G class I molecules (Schust et al 1998).

The Vpu protein of human immunodeficiency virus (HIV) also directs host proteins to their proteasome-dependent demise. HIV infection can result in the diminished cell surface expression of class I molecules, although the magnitude of this effect varies considerably depending on the host cells, virus strain, infection conditions, and antibodies used. Decreases in class I expression have been ascribed solely to the activity of Vpu (Kerkau et al 1997) or Nef (Schwartz et al 1996), based on results obtained with HIV engineered not to express the respective protein. Because both proteins, when expressed in isolation, have been shown to decrease class I expression, it is unclear why deletion of a single gene does not result in a partial phenotype. Although the physiological relevance remains to be established, the effects of Vpu and Nef on class I biogenesis are clearly of interest mechanistically.

Vpu is a 81-residue protein with a type II membrane anchor that is distributed throughout the secretory pathway. Vpu exhibits two distinct functions: degradation of host proteins and formation of an ion channel that enhances virus release (Schubert et al 1996). Vpu-induced degradation of the HIV coreceptor CD4 glycoprotein was one of the better-characterized examples of ERAD in the pre-proteasome inhibitor era. However, it was found that it too reflects proteasome-mediated destruction because it is blocked by proteasome inhibitors (Fujita et al 1997), requires a functional E1-activating enzyme, and can be blocked by mutating Lys residues in the CD4 cytosolic tail that are prime candidates for ubiquitination (Schubert et al 1998). As reported with several other ERAD substrates (Bonifacino & Weissman 1998), active proteasomes are required for the Vpu-induced export of CD4 to the cytosol, since incubation of cells with proteasome inhibitors results in the cell surface expression of CD4 rescued from degradation. This would appear to differ from US2/11, which continue to cytosolically translocate class I molecules in the presence of proteasome inhibitors. Whether this represents a genuine mechanistic difference or methodologically related differences remains to be resolved.

Vpu appears to treat class I molecules similarly to CD4. When expressed by rVV, Vpu reduced the amount of [^{35}S]-Met-labeled mouse or human class I molecules recovered by either antibodies specific for folded molecules or antibodies specific for unfolded heavy chains (Kerkau et al 1997). Class I molecules spared degradation were transported from the ER with normal kinetics, usually indicative of the possession of relatively high-affinity peptides. By contrast, there was no immediate reduction in the amount of class I molecules recovered from cells expressing VV-encoded ICP47, and molecules spared the slower destruction were not exported from the ER.

While these findings support the proteasome mediated destruction of class I molecules induced by Vpu, this remains to be established, as does (*a*) the binding of Vpu to class I molecules, (*b*) the basis for the recognition of two very different proteins (CD4 and class I molecules), and (*c*) the degree to which other proteins are affected by Vpu.

Another One Bites the Dust: Diverting Class I Molecules to Lysosomes The most recently described addition to the viral anti-class I armamentarium is mCMV gp48, a type I anchored integral membrane glycoprotein of 336 residues (Reusch et al 1999). gp48 is expressed within the first three hours of mCMV infection of tissue culture cells and is synthesized continuously thereafter. Its expression results in downregulation of each of seven mouse class I allomorphs tested. gp48 binds to class I molecules bearing endo H-sensitive oligosaccharides, indicating that binding occurs in the proximal regions of the secretory compartment, but details regarding the conformational state of class I molecules recognized and the compartment in which binding is initiated remain to be established. Class I molecules synthesized in the presence of gp48 acquire oligosaccharides characteristic of the *trans*-Golgi complex but are degraded with a $t_{1/2}$ of ~1 h. The degradation can be blocked by interfering with endosomal/lysosomal proteases via several approaches, which result in the accumulation of class I molecules and gp48 in lysosomes. Lysosomal targeting and degradation are also blocked by altering a di-Leu motif in the gp48 cytosolic tail. In this case class I molecules are simply routed to the cell surface while bound to the modified gp48. Di-Leu motifs are known to direct proteins to lysosomes, and it is always difficult to determine to what extent routing occurs directly from the *trans*-Golgi complex or via the cell surface. The failure to detect gp48 on the cell surfaces suggests the former but is also consistent with rapid internalization process. The capacity of gp48 to induce internalization of pre-existing class I molecules would demonstrate that routing from the plasma membrane can occur and would also provide important insight into the in vivo function of gp48. As discussed below, tools are now available for dissecting the mechanism of lysosomal routing.

Do That to Me One More Time: Internalization of Cell Surface Class I Molecules Nef is an N-terminal myristoylated 206-residue protein localized in both the cytosol and cellular membranes. Much like Vpu, the effect of Nef on cellular proteins

was first found with CD4, whose endocytosis is stimulated up to tenfold by Nef expression (Oldridge & Marsh 1998). The effect of Nef on class I expression was first examined by comparing the effects of Nef-expressing and non-expressing HIV during chronic infection of cells in culture (Schwartz et al 1996). This revealed that Nef expression does not affect the assembly or modification of class I molecules by the *trans*-Golgi complex but reduces the lifespan from a $t_{1/2}$ of 7 h to 4 h. Nef expression was associated with the accumulation of class I molecules in early endosomes, as determined by immunofluorescence. At least some of these molecules appeared to derive from the cell surface (as opposed to direct routing from the Golgi complex), as suggested by increased rate of internalization of antibodies bound to class I molecules (an approach that suffers from the perturbing effects of antibody cross-linking of class I molecules). The effects of interfering with the activity of endosomal/lysosomal proteases on Nef-mediated class I degradation by use of drugs that increase the pH of acid compartments or specifically block proteases remain to be examined.

These findings were extended by demonstrating that expression of Nef from a transfected gene reduced HLA A2 expression on the surface of HeLa cells (Le Gall et al 1998). This effect required the presence of the A2 cytoplasmic domain encoded by exon 6 (residues 314 to 324) and could be abrogated by mutating Tyr_{320} to Phe. Notably, HLA B alleles possess Tyr_{320}, whereas HLA C alleles do not, and a representative allele of each obeyed the Tyr_{320}-rule when tested by mutagenesis. These findings were correlated with immunofluorescence localization of A and B molecules, both of which were redistributed (in a Tyr_{320} -dependent manner) by Nef expression to colocalize with clathrin/γ-adaptin. The patterns of clathrin/γ-adaptin staining were not affected by Nef expression; both were present in small endosomes and the Golgi complex.

Additional findings using chimeric proteins, with Nef acting as the cytosolic domain, indicate that the Nef domain delays transport of the chimeras in the Golgi complex, which results in the accumulation of proteins at this site (Mangasarian et al 1997). On the other hand, Nef expression in human IMR90 fibroblasts resulted in the delivery of W6/32-class I complexes formed at the cells surface (or endosomes) to the Golgi complex (Greenberg et al 1998), thus indicating that Nef-induced accumulation of class I molecules in the Golgi complex can occur via multiple routes.

The mechanism of Nef-induced redistribution of class I molecules (and CD4) has also been investigated using the yeast two-hybrid system to identify cellular ligands of Nef, which revealed that Nef interacts with the μ components of the AP-1 and AP-2 complexes that associate with clathrin at clathrin-coated pits (Le Gall et al 1998). This interaction was confirmed by direct biochemical binding assays. The interaction of Nef with AP-1 appears to be more important for class I internalization, because mutant Nef molecules unable to interact with AP-2 (and thereby down-regulate CD4) still down-regulate class I molecules. Conversely, mutations in Nef that affect class I down-regulation do not affect CD4 (Greenberg et al 1998).

Because the cytoplasmic tail of class I molecules does not detectably interact with AP-1 or AP-2 by the same criteria (Greenberg et al 1998, Le Gall et al 1998), these findings suggest that Nef acts by enhancing the direct interaction between class I molecules and AP-1/AP-2, by bridging class I molecules to AP-1/AP-2 or by both mechanisms. Additionally, as both class I molecules and AP-1/AP-2 are phosphoproteins and Nef modifies cellular phosphorylation (Peter 1998), this may contribute to enhanced class I association with clathrin-coated pits.

The mechanism of Nef-mediated effects on class I is obviously intricate (it has also been reported that Nef binding to the major endosomal vacuolar ATPase is required for CD4 internalization; Lu et al 1998), and the effects may be further complicated by cell type-specific effects, as well as the modulating effects of other HIV proteins. The most important issue is the extent to which Nef acts by directly binding to class I molecules as opposed to indirect effects that affect multiple cell surface proteins. It is likely that unraveling the effects of Nef on protein internalization will require investigation for many years, as will trying to understand the role of Nef (and Vpu) in HIV infectivity and pathogenicity.

You've Really Got a Hold on Me: Binding to Cell Surface Class I Molecules gp34 is a mCMV type I-anchored integral membrane glycoprotein of 266 residues that tightly associates with mouse class I molecules in NP40 extracts (Kleijnen et al 1997). The effects of gp34 on class I biogenesis are largely based on the use of mCMV-infected cells, so the contribution of other viral proteins to functions ascribed to gp34 remains to be determined. gp34 binding to mouse class I molecules is allomorph specific and is dependent on expression of β_2m or TAP. This TAP dependence is partially overcome by expression of human β_2m, which suggests that gp34 binding depends on only class I molecules assuming the proper conformation and not on the formation of the TAP-class I complex. Binding occurs in the ER and is maintained throughout the secretory pathway so that gp34-class I complexes are stably expressed at the cell's surface and can be recovered following cell surface iodination. In fact, it appears that gp34 requires class I molecules to exit the ER since when an antiserum raised to a gp34-encoded peptide that is unable to bind to class I-gp34 complexes was used, the gp34 recovered was completely sensitive to endo H treatment. Moreover, as gp34 expression is associated with increased class I export from the ER, it appears that gp34 alleviates the class I blockade effected by mCMV gene products expressed earlier in the infectious cycle.

The next chapter in this fascinating story will concern the function of gp34-class I complexes. It is likely that these molecules serve to confound the efforts of NK cells. If so, there must be a mechanism for preventing T_{CD8+} recognition of gp34-class I complexes. To wit, the complexes may lack peptides, with gp34 providing the necessary stabilizing influence to prevent class I denaturation, or gp34 may block TCR recognition of class I peptide complexes.

I Will Survive: Temporal Control and Multigenic Effects Thus far we have largely ignored two issues that are critical to viral interference with antigen

processing: temporal expression of modulatory proteins and synergistic effects of multiple gene products. This aspect of viral interference is best illustrated by hCMV, which as discussed above, expresses at least five gene products, pp65, US2, US3, US6, and US11, that interfere with antigen processing. Current understanding suggests the following scenario.

1. Viral cores are delivered to the cytosol initiating the infectious cycle.
2. Viral gene expression begins with expression of IE products, and pp65 prevents presentation of the most abundant gene product IE.
3. One of the IE gene products expressed is US3, which is synthesized from 1 h post-infection (pi) to 4 h pi. US3 retains class I molecules in the ER containing peptides from IE molecules.
4. Then US2 and US11, synthesized starting at 5–6 h pi, degrade class I molecules retained by US3 as well as more recently synthesized class I molecules.
5. These effects are enhanced by the TAP blockade exerted by US6, whose expression begins at 6 h pi but peaks much later.

The obvious question is why such a complicated mechanism is required? There are a number of potential answers (whose airing also serves to place the actions of all of various viral inhibitory proteins in perspective). First, the redundancy may be needed since any single mechanism may miss too many allomorphs. Second, the proteins may have different efficacies in different tissue types infected by the virus. Third, the virus may be gingerly negotiating T_{CD8+}-NK recognition, titrating antigen presentation based on the preponderance of NK cells early in the infection and that of T_{CD8+} later on. Finally, there are surely some things we humans are just not meant to know.

Sounds of Silence: Roads (Apparently) Not Taken It now should be clear that viruses have devised numerous strategies to interfere with antigen processing. Given the potential for viruses to hijack and modify host genes, it is likely that the absence of a potential mechanism for interfering with antigen presentation reflects too high a cost in viral replication and not an inability to accomplish the task.

There are two strategies whose absences are particularly conspicuous. The first is a general blockade of proteasome activity (achievable even by the lowly bacterium that produces lactacystin). This absence suggests one or both of the following. (*a*) The only means of blocking proteasomes compromises their activity to such an extent that cell metabolism is affected and viral replication suffers. (*b*) There are too many alternative means of producing antigenic peptides to gain a selective advantage.

The second is the sequestration or destruction of β_2m. Knocking out β_2m expression has a profound impact on antigen presentation, not complete, but much greater than blocking or knocking out TAP, for example. It is possible that β_2m

is targeted by an undiscovered viral gene product. On the other hand, why should *Herpesviridae* evolve ten or more distinct gene products to interfere with antigen presentation when a single gene product that inactivates β_2m would accomplish the same task? One answer is that β_2m expression is also required for proteins needed to support viral replication or latency. A candidate gene product is HFE, the nonclassical class I molecule involved in iron metabolism. However, tumor cells frequently delete β_2m, which argues persuasively that any changes in cellular metabolism associated with the loss of β_2m can be accommodated by viruses. Another answer, and probably the correct one, is that the decrease in class I expression would present too easy a target for NK cells.

FUTURE CHALLENGES

Many challenges remain for those investigating viral subversion of antigen presentation, a field poised for explosive growth. Some of the most important challenges are

1. Establishing a more detailed mechanistic understanding of how each given viral gene product described in this review works. The recent demonstration that adenovirus E19 managed to keep a trick or two up its sleeve for 20 + years after its discovery warns against the risks of intellectual complacency. There are considerable opportunities for X-ray crystallographers to provide the structural basis for the interaction between class I molecules and their viral protagonists.
2. The discovery of new viral molecules and new cellular targets for viral molecules. The most promising sources of new viral genes are the large DNA viruses that chronically infect hosts, but given that exceptions are the rule in biology, even those studying small RNA viruses with hit-and-run replication strategies should be on the lookout for interference with antigen processing.
3. Coming to grips with the overall effect of viral replication on antigen presentation in vivo. When multiple viral gene products are involved in modulating class I presentation, how do they work in concert? Why do different viruses choose different strategies; for example why is it that hCMV blocks early events in class I presentation (blocking TAP) while mCMV concentrates on interfering with later events in class I presentation? What role does NK cell recognition have on these strategies? Does the allomorph specificity exhibited by viral gene products reflect an evolutionary strategy of the virus or the host?
4. Practical application of knowledge of viral interference with antigen presentation toward producing more immunogenic live viral vaccines and blocking immune recognition in gene therapy, transplantation, and autoimmunity.

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